AD_____

Award Number: DAMD17-03-1-0445

TITLE: Molecular Identification of the Schwannomatosis Locus

PRINCIPAL INVESTIGATOR: Mia MacCollin, M.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Charlestown, MA 02129

REPORT DATE: July 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2007	Annual	1 Jul 2006 – 30 Jun 2007

4. TITLE AND SUBTITLE

Molecular Identification of the Schwannomatosis Locus

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0445

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Mia MacCollin, M.D.

Email: maccolin@helix.mgh.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
Charlestown, MA 02129

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Schwannomatosis is a recently recognized third major type of neurofibromatosis. Our preliminary studies of the NF2 gene in tumors from schwannomatosis patients reveal a pattern of tumor suppressor gene inactivation not previously reported in any other human disease. **Objective/Hypothesis:** Th objective of this project is to clone the locus responsible for familial schwannomatosis. We are exploring two competing hypotheses which address both the non random distribution of LOH observed in schwannomatosi tumors and the high rate of somatic NF2 mutation seen along the cis allele. **Specific Aims:** 1. To identify and clinically characterize schwannomatosis patients, and maintain a resource of tumor an blood specimens. 2. To further refine the candidate region on chromosome 22 using linkage and loss of heterozygosity analyses. 3. To determine the molecular mechanism of tumor formation in these patients using complementary molecular and cytogenetic approaches. **Study Design:** Schwannomatosis patients and affected relatives will be identified. Blood and tumor specimens will be obtained f linkage, LOH, FISH and mutational analysis of coding and non coding candidate regions. **Relevance:** This study will elucidate t unique pathogenesis of schwannomatosis and provide a means for definitive diagnosis using molecular technology.

15. SUBJECT TERMS
schwannomatosis, tumor suppressor gene, NF2, molecular genetics

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Neurofibromatosis (NF) encompasses a diverse group of genetic conditions whose common element is tumors of the nerve sheath. Schwannomatosis is a recently recognized third major type of NF, which results in multiple schwannomas without vestibular tumors diagnostic of NF2. Recent epidemiological studies have shown that schwannomatosis is as common as NF2. Our preliminary studies of the *NF2* gene in tumors from schwannomatosis patients revealed a pattern of tumor suppressor gene inactivation not previously reported in any other human disease. The objective of this project is to clone the locus responsible for familial schwannomatosis. We are exploring two competing hypotheses which address both the non random distribution of LOH observed in schwannomatosis tumors and the high rate of somatic *NF2* mutation seen along the *cis* allele. The first hypothesis is that schwannomatosis is due to mutation in a second tumor suppressor gene which lies near *NF2* on chromosome 22. In this model, schwannoma formation is dependent on four "hits" (two in the schwannomatosis tumor suppressor, and two in the linked *NF2* tumor suppressor). FISH results have suggested a second hypothesis in which a structural element facilitates loss of the *trans* chromosome by increasing the rate of mitotic recombination. This is an especially attractive hypothesis since rates of mitotic recombination are both highly variable and genetically determined in humans.

Body

This section is organized around the approved statement of work.

Task 1. To develop a resource of study subjects and related biological materials (months 1 through 30): Task completed at time of last report.

Task 2. To refine the candidate region using LOH and linkage analysis (months 12 through 36): Task completed at the time of last report

Task 3. To determine the molecular mechanism leading to schwannomatosis (months 1 through 48):

Subtasks a through d completed at time of last report.

e. Screening of candidate regions and/or loci based on results of task 2 (months 30 through 45). Recently, mutations in the *SMARCB1* tumor suppressor (also known as *INI1* and *hSNF5*), which lies in the familial schwannomatosis candidate region, were detected on a somatic and constitutional level in a single kindred with schwannomatosis (Hulsebos et al., 2007). The protein product of the *SMARCB1* locus is part of the human transcription complex and was cloned as the human homolog of yeast complex member *SNF5* in 1994 (Kalpana et al., 1994). Although it was originally identified as the binding partner for HIV-1 integrase (and was thus called *INI1),* in 1998 Versteege et al. mapped overlapping deletions in pediatric malignant rhabdoid tumors and found the smallest region of overlap was the *hSNF5* gene. In addition, point mutations were detected as the first hits in cell lines confirming that the molecule was a true tumor suppressor for this tumor type.

In addition to these constitutional changes, at least 27 unrelated individuals and 5 unrelated families have been reported in the literature with constitutional truncating mutations in the *SMARCB1* gene (for example, Biegel et al., 1999). All have rhabdoid tumor, although some family members have also developed other tumor types (choroid plexus carcinoma, medulloblastoma and PNET although there is some controversy in the literature regarding pathological misdiagnosis). Three of the families consist of siblings with genetically normal parents implying germline mosaicism, while the other two consist of families with nonexpressing adult carriers. Until the report of Hulsebos et al., no patient with a constitutional *SMARCB1* change was know to have schwannomas or other nerve

sheath tumors. This body of work is additional proof that *SMARCB1* is a true tumor suppressor, albeit in a syndrome which lacks nearly any clinical relationship to schwannomatosis. We therefore sought to determine how generalizable the findings of Husebos et al. might be and specifically to determine how frequently *SMARCB1* was a tumor suppressor for schwannomas in the context of familial schwannomatosis..

We are studying 19 schwannomatosis kindreds who were unrelated to the best of the study participants' knowledge. Genomic DNA from 6 non founder EBV transformed cell lines, 12 schwannomas previously characterized at *NF2* and one meningioma in short term culture were used for the primary screen. The intron exon structure of the *SMARCB1* transcript was determined by comparison of the cDNA sequence (accession number AB017523) with the genomic sequence (AP000349 and AP000350). Primers were designed to amplify a minimum of 25 basepairs of flanking intronic sequence on either side of each exon. In addition, primers for exon 4 were designed to amplify the 51 basepairs included in the rare alternatively spliced form identified by two investigators (Mimori et al., 2002 and pers. com., Favre et al., 2003). Initial unidirectional automated sequencing of PCR products was performed on a single sample from each kindred; areas of poor sequencing, ambiguous or definite change were sequenced from the opposite direction or from primers internal to the amplification primers. Confirmed alterations found in tumor samples were immediately assessed in paired blood samples; alterations in blood samples were then assessed in affected and unaffected relatives. Because the *SMARCB1* locus is especially rich in polymorphism, all alterations were assessed in the single nucleotide polymorphism (SNP) database accessed through the NCBI website (http://www.ncbi.nlm.nih.gov/projects/SNP/). In addition to mutational analysis of the *SMARCB1* locus, we used microsatelite markers developed in previous years of funding to determine potential loss of heterozygosity of the region and potential identity by descent when identical changes were found in two or more families. Tumors entered in this screen had previously been analyzed at the *NF2* locus for both mutation and loss of heterozygosity as described in previous years' progress reports.

Thus far we have detected no unreported alterations in exons 5, 7 and 8. Informativeness at known SNPs have been detected in a small percentage of samples in exons 5 (rs5751738), 6 (rs2070457), 7 (rs35817983 and rs2229354 which appear to be in linkage disequilibrium), and 9 (rs34399789). In exon 5, rs5760030 is GG or G/- in all samples rather then A as in the database. Three changes which are unreported in the SNP database but are unlikely to be pathogenic were seen in exons 1 (c93+105GC(7_9) and c.93+121_93+122dup(TC)) and 3 (c. T233-43A on an unaffected allele). Two changes of unclear pathogenicity were seen. In exon 6, a single family is segregating c.629-5T>G on the affected allele. Although this position is not highly conserved in the mammalian splice acceptor, it may in fact influence splicing weakly. We are currently assessing its presence in a panel of 100 unrelated unaffected alleles and its effect on splicing in mRNA derived from this kindred. In the 3' untranslated region (exon 9) c.1240C>T is present in family 1 (unphased), and on the affected alleles of family 3, family 5 and family 10. We are currently assessing the possibility of identity by descent of these four kindreds by determining the shared alleles at the internal polymorphisms described above and at microsatellites flanking *SMARCB1* developed in previous years' funding.

We have detected two non truncating, potential missense mutations in three kindreds. In exon 1 the change c.41C>A, p.Pro14His was seen in a tumor from family 4. There was no loss of heterozygosity in this tumor and the change was present in the paired blood sample and in other affected family members. This is a non conservative amino acid change which is identical to the level of *drosophila*. We have assessed the alteration in a panel of 50 unaffected unrelated individuals and have not detected it in those 100 alleles. A second missense alteration was found in exon 2,

c.158G>T, p.Arg53Leu. This change was seen in tumors from both family 9 and family PA-1 and the corresponding blood samples. These two families have no known relationship, but neither family can identify a founder and they live in geographic proximity. Loss of heterozygosity of the alteration was seen in both tumors compared to the paired blood samples, and the change was present in all affected family members examined. This is also a non conservative amino acid change which is identical to the level of *xenopus*. We have assessed this alteration in a panel of 50 unaffected unrelated individuals and have not detected it in those 100 alleles. Subsequently, we have examined microsatellite markers in these two families and determined that they most likely have identity by decent and thus an unknown shared founder (table 1).

	centromeric				telomeric			
Family	D303 21,605	AB10 21,853	AB02 22,306	AB05 22,380	AB09 22,769	AB08 22,819	AB03 22,840	D1174 22,818
PA-1	223	R, 179, 185	135	160	225	341	201	196
9	225	179	135	160	225	341	201	196
Control			132	167	217			198
Control			132	157	211			
Control			135	160	215			

Table 1. Retained and presumably affected alleles in tumor material. Microsatellite markers are classified as centromeric or telomeric to the *SMARCB1* locus itself (at 22,459,150 (5'UT) to 22,506,705 (3'UT) in build 36.2). Absolute position of markers in kilobasepairs is given below the marker name. Families PA-1 and 9 share an affected allele at all markers except D22S303, making their descent from a common ancestor likely. An unseen crossover event has presumably separated the D22S303 locus from the causative mutation in an intervening mitosis. The 135-160-225 haplotype defining the region most closely linked to *SMARCB1* is not seen in three control affected alleles from other families.

We have detected three mutations in conserved splice donor sites. In exon 3 c.362+1G>A was found in a tumor from family E. There was no clear loss of heterozygosity and the alteration was present in an affected family member. In exon 4 c.500G>A was seen at the ultimate basepair of exon 4. This change was present in all affected family members sequenced. This alteration of a highly conserved splice donor position could have multiple effects. If it encouraged a greater expression of the alternatively spliced "long form" of exon 4 an inframe stop codon would be produced (p.Trp167X). If splicing was maintained to exon 5, a missense change would be produced (p.Cys167Tyr). We are currently assessing cDNA from this family to see which alterative is more prevalent. The second splice donor alteration was seen in exon 6. c.795+1G>T was detected in a blood sample and we are currently pending assessment in affected relatives.

Finally, and perhaps most surprisingly, we have detected two nonsense mutations in exon 4 and the rare long isoform of that exon. In family 11, c.364G>T, p.Glu122X was present with loss of heterozygosity in a tumor specimen, the paired blood sample and all affected relatives. In family V, c.500+5G>T p.Gly167+2X was present with loss of heterozygosity from a tumor specimen, the paired blood sample and all affected relatives. A summary of mutational analysis combining the results described in this progress report with previous results obtained at the *NF2* locus in these samples is presented in table 2.

	NF2 locus				*SMARCB1* locus			
Family ID	Exon	Sequence	Effect	LOH	Exon	Sequence	Effect	LOH
1	NA				none			NA
3	3	c.241-3_260del	SP	Y	none			Y
4	10	c.979delG	FS	N	1	c.41C>A	MS	N
5	7	c.630_631insT	FS	Y	none			Y
8	NA				none			NA
9	none			N		c.158G>T	MS	Y
10	NA				none			NA
11	none			R	4	c.364G>T	SP	Y
NH	2	c.179G>A	NS	Y	none			Y
Mo	none			N	none			N
E	7	c.600-1G>C	SP	Y	3	c.362+1G>A	SP	Y
PA-1	8	c.778_782del	FS	Y	4	c.158G>T	MS	Y
PA-2	11	c1021C>T	NS	N	none			N
PA-3	NA				4	c500G>A	SP	NA
P/Qu	none			Y	6	c.629-5T>G	?SP	N
V	6	c.551G>A	NS	Y	4	c.500+5G>T	NS	Y
SD	NA				none			NA
18	6	c.596delC	FS	Y	none			Y
19	NA					c.795+1G>T	SP	NA

Table 2. Comparison of previous *NF2* mutational results to *SMARCB1* analysis. All *NF2* gene mutations are somatic (in tumor tissue; not present in blood) and all *SMARCB1* gene alterations are constitutional. Unlike the previous results of Hulsebos et al., *SMARCB1* mutations were detected in tumors which also bore *NF2* gene mutations. Predicted effect on the protein product is given as SP—splice site alteration, FS—frameshift, NS—nonsense mutation, MS—missense. LOH—loss of heterozygosity of surrounding microsatellite markers in tumor compared to blood. NA—no tumor available for analysis

These findings raise an obvious question of why mutations in the same transcript can produce widely differing phenotypes of childhood atypical teratoid/rhabdoid tumor (AT/RT) and schwannomatosis. To date, a total of 32 constitutional mutations have been reported in 27 unrelated patients with AT/RT and 5 unrelated families segregating AT/RT. These alterations are composed of 17 nonsense, 14 frameshift and a single splice site mutation in exon 7. Constitutional mutations have not been reported in exons 1, 8 and 9, although exon 9 is the most frequently altered in a somatic setting. Mutations have also not been reported in the alternatively spliced long form of exon 4 although it is unclear from most reports if this area was actually screened for changes. Our results suggest some clear difference in both type (with over representation of missense and splice and under representation of nonsense and frameshift) and location (with involvement of exon 1) of alteration in schwannomatosis versus AT/RT but we are currently pending a more rigorous meta analysis of the literature to determine the statistical significance of this impression.

Key Research Accomplishments

Development and validation of screening protocol for small alterations in the *SMARCB1*

gene.

Detection of polymorphisms useful for further studies of identify by descent and expression.

Confirmation of the hypothesis that *SMARCB1* is a tumor suppressor for schwannomas in the context of familial schwannomatosis.

Preliminary identification of fundamental differences between *SMARCB1* mutations causing the phenotype of AT/RT and those causing schwannomatosis.

Reportable Outcomes

Presentations:

Invited presentations by the PI concerning this research were made to the Department of Biochemistry and Biophysics symposium, Oregon State University, Corvallis, OR 11/06, to the Washington State Neurofibromatosis Foundation 12/06 and to the Neurofibromatosis Mini Symposium, Heerlen, The Netherlands, 4/07.

An invited presentation by Ms. Chelsea Boyd concerning this research was made to the Children's Tumor Foundation, Park City, Utah (6/07).

Funding applied for:

The PI supported the submission of a proposal titled "Molecular characterization of *SMARCB*1 in familial and sporadic schwannomas, " (PI Scott Plotkin) to the NIH in June, 2007.

Research opportunities:

The PI sponsored Dr. Miriam Smith as a postdoctoral fellow in the Department of Neurology at Mass General Hospital, 5/07 to present.

Conclusions

Schwannomatosis is a third major form of NF, which recent epidemiological studies have shown is as common as NF2. However, clinical recognition and molecular characterization have lagged far behind other forms of NF. The clarification of molecular alterations in schwannomatosis will likely have broad implications for other tumor suppressor gene syndromes. We have identified alterations in the *SMARCB1* gene over the past year which confirm that it is a tumor suppressor for familial schwannomatosis. Further work is needed to determine its role in other multiple and single tumor syndromes.

References

Biegel JA, Zhou JY, Rorke LB, Stenstrom C, Wainwright LM, Fogelgren B. Germ-line and acquired mutations of INI1 in atypical teratoid and rhabdoid tumors.
Cancer Res. 1999 Jan 1;59(1):74-9.

Favre M, Butticaz C, Stevenson B, Jongeneel CV, Telenti A. High frequency of alternative splicing of human genes participating in the HIV-1 life cycle. J Acquir Immune Defic Syndr 2003;34:127-133.

Hulsebos TJ et al., Germline mutation of *INI1/SMARCB1* in familial schwannomatosis. Am J Hum Genet 2007;80:805-10.

International HapMap Consortium. A haplotype map of the human genome. Nature 437: 1299 1320 (2005), data accessed at: http://www.hapmap.org/

Kalpana GV, Marmon S, Wang W, Crabtree GR, Goff SP. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science 1994:266:2002-6.

Mimori K, Inoue H, Shiraishi T, Ueo H, Mafune K, Tanaka Y, Mori M. A single-nucleotide polymorphism of SMARCB1 in human breast cancers. Genomics. 2002 Sep;80(3):254-8.
Versteege I, Sevenet N, Lange J, Rousseau-Merck MF, Ambros P, Handgretinger R, Aurias A, Delattre O. Truncating mmutations of *hSNF5/INI1* in aggressive paediatric cancer. Nature 1998;394:203-6.